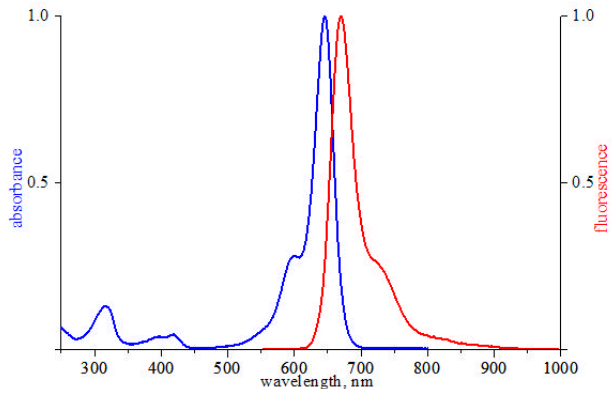
DATA SHEET

Jena Bioscience

Atto 647N Protein Labeling Kit

Fluorescent labeling of primary amino groups

Cat. No.	Amount
FP-201-647N	10 reactions



excitation and emission spectrum of ATTO 647N

For general laboratory use.

Shipping: shipped on gel packs

Storage Conditions: store at -20 °C

Additional Storage Conditions: store dark, see manual

Shelf Life: 12 months

Spectroscopic Properties:
Excitation maximum: λ_{exc} = 644 nm
Emission maximum: λ_{em} = 669 nm
Extinction coefficient: ε_{max} = 150,000 mol^{-1} cm^{-1}
Correction factor: $CF_{280\ nm}$ = 0.05

Description:
Fluorescence techniques have become a major tool in biological sciences. Fluorescent proteins such as GFP and DsRed fused to the protein of interest (POI) allow expression analysis and in vivo protein localization. Their application however, is limited due to the large molecular weight of those fusion proteins. Furthermore, the associated molecular biology is tedious and time-consuming.
Small fluorophores, covalently attached to the POI, may help to overcome this problem. Fluorescent labeling of the thiol group of cysteines is preferred for investigating structure, function and interactions of the POI. (Please note: Jena Bioscience offers a ready-to-use kit for labeling of cysteine residues with high-quality small fluorophores (Cat.# FP-202) for application in protein activity and functional studies).
The most common labeling method for proteins however, is amine modification. Amine-reactive fluorophores such as NHS-esters readily react with primary amino groups present at the N-terminus[1] as well as in lysine side chains, resulting in a stable covalent bond.
This Jena Bioscience Protein Labeling Kit is designed for labeling the lysines of a POI with a small fluorophore resulting in a fluorescent protein-fluorophore conjugate. It contains all reagents required for performing 10 separate labeling reactions of 1 mg of POI.

Content:
Atto 647N NHS-ester
1 vial containing 1 mg

Dimethylformamide (DMF)
200 µl

Sodium bicarbonate
1 vial containing 84 mg

ultra-pure water
1 ml

Storage and Stability:
Upon receipt store the dye at -20 °C. The other components may be stored at room temperature. If stored as recommended, Jena Bioscience guarantees optimal performance of this product for 12 months.

Protocol:
General notes
The optimal protein concentration is 10 mg/ml, other concentrations are also possible, however, it should be at least 2 mg/ml since labeling efficiency suffers from lower protein concentrations. We recommend using about 1 mg protein per labeling reaction.
Buffers containing primary amines such as Tris and glycine are not suitable for the labeling reaction and must be exchanged with suitable amine-free buffer such as PBS, MES, or HEPES before starting the labeling reation.

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Experimental protocol

- Dissolve the sodium bicarbonate by adding 1 ml ultra-pure water. The resulting 1 M solution is stable at 4 °C for at least 2 weeks.
- Add the appropriate volume of sodium bicarbonate (1 M) to your protein solution to achieve a final concentration of 100 mM.
- Prepare the dye by adding 100 µl DMF resulting in a dye concentration of 10 mg/ml. Vortex until the NHS ester is completely dissolved! We recommend preparing the solution shortly prior to use however, it is stable for two weeks at -20 °C. Whenever handling the fluorophore or conjugate, work under low light conditions!
- Add 100 µl protein solution (10 mg/ml) and 10 µl dye (10 mg/ml) to an appropriate vial. Vortex carefully and centrifuge briefly to collect the reaction mixture at the bottom of the tube.
- Incubate for one hour in a shaker at room temperature. Protect from light!
- Purify the conjugate using standard gel filtration columns such as Sephadex G-25 or similar. Alternatively, the free dye may be separated from the conjugate by dialysis or appropriate spin concentrators.

Please note that protein purification materials are not provided with the kit!

Concentration of the conjugate

Since there may occur a certain loss of protein during labeling and particularly during purification measuring the concentration of the conjugate is important for further application. The concentration of a protein is commonly determined by measuring its absorbance at 280 nm. As shown in the excitation spectrum of Atto 647N however, the fluorescent dye absorbs at 280 nm as well thereby increasing the ε_{280} for the conjugate. Therefore, a correction factor (CF) is required to eliminate the contribution of the dye at 280 nm.
The CF of each dye is given in the Spectroscopic Data section, and hence the concentration of the conjugate is calculated according to:

$$c\ (mg/ml) = ((A_{280} - A_{max} \times CF) / \varepsilon_{280}) \times MW_{protein}$$

A_{280}	absorbance of the conjugate solution measured at 280 nm
A_{max}	absorbance of the conjugate solution measured at λ_{exc}
λ_{exc}, ε_{max}, CF	intrinsic properties of the Atto dye, please refer to Spectroscopic Prop
ε_{280}, MW	intrinsic properties of your protein, if not known they can be obtained from web sources such as ExPASy Proteomics Server[2]

Degree of labeling (DOL)

The DOL specifies the average number of fluorophore molecules per molecule of conjugate. It is an important parameter of the conjugate significantly affecting further applications. For most purposes, a DOL of approximately 1 molecule of fluorophore per 200 amino acids is ideal, also see section Troubleshooting.
The DOL is calculated according to:

$$DOL = (A_{max} \times \varepsilon_{280}) / ((A_{280} - A_{max} \times CF) \times \varepsilon_{max})$$

Example for determining the conjugate concentration and the DOL of BSA (Bovine serum albumin), labeled with Atto 488 NHS ester:
BSA: ε_{280} = 42,925 M^{-1} cm^{-1}, MW = 66,433 Da
Atto 488: λ_{exc} = 501 nm, ε_{max} = 90,000 M^{-1} cm^{-1}, CF = 0.1

After labeling and purification the absorption of the conjugate solution is measured at 280 and 501 nm, respectively.

-	absorption
A_{280}	1.69
A_{501}	7.23

The conjugate concentration and the DOL are calculated:

$$c\ (mg/ml) = ((A_{280} - A_{max} \times CF) / \varepsilon_{280}) \times MW_{protein}$$
$$c\ (mg/ml) = ((1.69 - 7.23 \times 0.1) / 42{,}925) \times 66{,}433$$
$$c\ (mg/ml) = 1.5$$

$$DOL = (A_{max} \times \varepsilon_{280}) / ((A_{280} - A_{max} \times CF) \times \varepsilon_{max})$$
$$DOL = (7.23 \times 42{,}925) / ((1.69 - 7.23 \times 0.1) \times 90{,}000)$$
$$DOL = 3.57$$

In this experiment the concentration of the conjugate is 1.5 mg/ml and most protein molecules are labeled with three or four Atto 488 molecules corresponding to 1.2 molecules of Atto 488 per 200 amino acids.
Please note that the spectroscopic determination of the concentration and the DOL of the conjugate is not absolutely correct. The spectroscopic characteristics of the free dye are not exactly the same as of the dye bound to the POI. Also, the spectroscopic characteristics of the native protein at 280 nm differ from those of the conjugate.
In general, these changes are negligibly small and therefore, spectroscopic determination of the concentration and the DOL of the conjugate is the most frequently used method[3] .
In addition to the spectroscopic measurements, one may analyze the conjugate by SDS-PAGE and subsequent fluorescence scanning of the gel. Only one band (consisting of the fluorescently labeled protein) should be visible in the fluorescent scan. If there is an additional band at very low molecular weight, the conjugate solution still contains free dye and must be purified again.

Storage of the Conjugate

Protect from light! Store the conjugate just like the unlabeled protein.

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We recommend dividing the solution into small aliquots and freeze at -20 °C or -80 °C. Avoid repeated freezing and thawing!

Troubleshooting:

Inefficient Labeling

- Concentration of protein solution
  The assay is optimized for labeling of 1 mg protein at a concentration of 10 mg/ml.
  Increase the amount of dye proportionally for protein concentrations above 10 mg/ml. If your protein concentration is very low (< 1 mg/ml) apply spin concentrators to achieve a final concentration of 2 mg/ml.
  The efficiency of labeling is strongly concentration dependent and varies among different proteins. Thus, in every single case optimization might be necessary to obtain the desired degree of labeling.
- Buffer composition
  Protein solutions containing primary amines (even traces thereof) dramatically decrease labeling efficiency. Make sure that your protein is extensively dialyzed in case it has been in contact with amine-containing substances.
- Impact of the pH
  Check the pH of your protein solution! The reference range is 8.2 - 8.5.
  The primary amino groups of the protein must not be protonated to be reactive thus, the pH of the protein solution has to be sufficiently high. On the other hand, the hydrolysis rate of NHS esters increases with the pH of the solution, resulting in non-reactive dye. Optimal labeling results have been obtained at pH 8.3.
  100 mM sodium bicarbonate might not be sufficient to raise the pH to optimum in strongly buffered protein solutions at a lower pH. You may add more sodium bicarbonate until the optimal pH is achieved.

Note that labeling efficiency not only depends on the surrounding conditions but also on the protein characteristics. The tertiary structure and the resulting number of lysines on the surface of the protein play a role as well as the isoelectrical point and the behaviour of the protein at pH 8.3.

Overlabeling

Reasons for overlabeling can be a high number of lysines on the surface of your protein and/or optimal characteristics of the protein at pH 8.3.
To prevent overlabeling, decrease the amount of dye or increase the protein concentration. You may also reduce the reaction time.

Purification of the conjugate

The dye is unstable in aqueous solutions - the hydrolysis rate of NHS esters increases with the pH of the solution. Thus, a certain amount of free dye is produced in each labeling reaction and needs to be separated from the conjugate.

If your purified conjugate still contains traces of free dye apply it to a second purification step. Check the purity of your conjugate by SDS-PAGE.
Separation of free dye from the conjugate becomes more difficult at higher concentrations of free dye. The column or membrane might even be blocked by the dye at very high concentrations, so try to optimize the labeling reaction in order to decrease the concentration of free dye. Keep in mind that overall yield suffers from additional purification steps.

What DOL is optimal?

As a basic rule one dye per 200 amino acids is ideal however, the optimal degree of labeling depends on your protein as well as on your intended application. If you are not sure, check your assay with small amounts of conjugate at different DOLs to obtain optimal results.
In any case prevent overlabeling since this may cause protein aggregation or fluorescence quenching of neighbouring dyes.

Selected References:

[1] The α-amino group at the protein's N-terminus may be labeled selectively by performing the labeling reaction at pH 7.0

[2] www.expasy.ch/tools/protparam.html

[3] Brinkley (1992) A Brief Survey of Methods for Preparing Protein Conjugates with Dyes, Haptens, and Cross-Linking Reagents. *Bioconjugate Chem.* **3**:2.